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# MELANOCYTE MIGRATION IN THE GUINEA PIG

## A Study of the Relationship of Melanocyte Population Density to Pigment Spread in Inoculation Grafts

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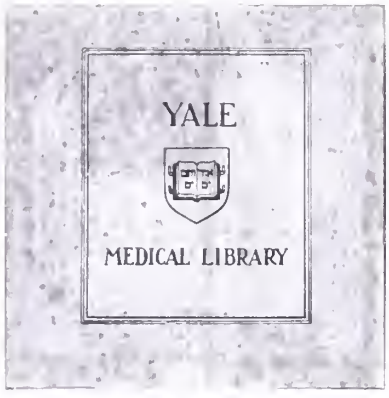
Class of 1982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# MELANOCYTE MIGRATION IN THE GUINA PIG:

## A Study of the Relationship of Melanocyte Population Density to Pigment Spread in Inoculation Grafts

by Katalin Eve Roth, B.A., J.D.
Yale University School of Medicine
Class of 1982

A Thesis Submitted to the Yale University
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of the Requirements for the Degree of
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To My Parents

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ABSTRACT

Inoculation grafts of epidermal cell suspensions were used to study melanocyte migration in juvenile piebald guinea pigs *in vivo*. Epidermal cell suspensions obtained from black ears were injected into suction blisters raised over achromic patches in autologous animals. Cell concentrations ranging from $2 \times 10^7$ and $5 \times 10^5$ cells/ml were tested. Pigment spots spreading from the graft sites were measured at two-week intervals. Skin biopsies for light and electron microscopy were taken at 7,55, and 90 days post grafting. Successful establishment of pigmentation was directly proportional to the concentration of pigment cells inoculated. Once a graft was established, the concentration of the inoculum did not significantly affect the area of pigment spread. At the end of 90 days, pigmented spots covered areas as large as 155 $mm^2$.

The density of melanocytes within the grafts was found to be approximately the same irrespective of the number of cells injected. Under light microscopy, the graft sites appeared identical to normally pigmented trunk skin. Melanocytes at the periphery of the graft sites were larger and more dendritic than those in the central regions. Electron microscopy showed grafted melanocytes to be adjacent to but not in contact with the basement membrane. Melanosomes within keratinocytes were clustered below the nuclei rather than in a supranuclear position.

We conclude that inoculation grafts of epidermal cells produce permanent, normally pigmented spots in achromic guinea pig skin and that the proportion of successful grafts is directly correlated with the concentration of melanocytes injected.

INTRODUCTION

Melanocytes have been shown to migrate in embryonic tissue (1), in mature epidermis (2) and also *in vitro* (3). One aspect of melanocyte locomotion in vivo, the phenomenon of pigment spread -- the centrifugal drift of dark coloration out from a black graft placed on achromic skin -- has intrigued biologists since it was first described in 1896 (4), but its mechanism remains incompletely understood. The present study was undertaken to examine whether local melanocyte population density influences pigment cell movement.

Inoculation grafts of melanocytes were utilized to study pigment cell migration in juvenile piebald guinea pigs. Epidermal cell suspensions of autologous melanocytes and keratinocytes in varying concentrations were injected into suction blisters raised on unpigmented trunk skin. Five cell concentrations ranging from $2 \times 10^7$ cells/ml to $5 \times 10^5$ cells/ml were tested and the results compared.

## BACKGROUND

### The Phenomenon of Pigment Spread

The phenomenon of pigment spread occurs in a variety of mammalian species, including man, but it has been most extensively studied in the guinea pig. Pigment spread occurs naturally to a limited degree: the migratory activity of pigment cells is necessary to the repigmentation of areas of skin in which melanocytes have been lost through trauma or disease. However, in clinical hypopigmentary situations areas of pigment loss remain stable. In some spotted animals pigment spread occurs as part of normal growth: for example, in piebald guinea pigs at birth the areas of pigmented skin, bearing black hairs, are sharply demarcated from white skin bearing white hairs; but as the animals mature there is progressive encroachment of pigment into the adjacent white epidermis, although the hairs in these transitional zones remain unpigmented (5). Artificial introduction of pigment cells into nonpigmented epidermis by means of skin grafts or inoculation grafts enhances the rate of this natural pigment spread, facilitating experimental analysis.

The earliest experimental studies of the pigment spread phenomenon, later recognized as a form of pigment cell migration, were carried out in 1896 by Carnot and Deflandre (4). These and subsequent investigators (6,7) found that when black skin from a piebald guinea pig is grafted to white skin in the same animal, the white skin surrounding the graft blackens from the graft margin radially outward. When white skin is grafted to a black area, the white graft blackens from the periphery

inward. And when black skin is grafted to red skin in tricolor animals, the red skin surrounding the graft blackens.

For many years the favored explanation for this phenomenon was the "infective hypothesis". The infective hypothesis postulated the existence of nonfunctional amelanotic melanocytes in white skin, which were thought to be "turned on" by some enzyme or infective agent transmitted from the black grafts to the surrounding white skin (7). Similarly, the pheomelanin-producing melanocytes of red skin were thought to be transformed by the infective agent into melanin-producing melanocytes of black skin. The infective hypothesis had two drawbacks: all attempts to initiate pigment spread with cell-free extracts introduced into white skin failed to produce pigmented epidermis; and the existence of the postulated amelanotic melanocytes was never demonstrated in the white skin of piebald animals.

In 1963 Billingham and Silvers, in a series of elegant experiments which utilized immunologically marked melanocytes in sequential grafts on hybrid strains of piebald guinea pigs, proved that the biologic basis of the pigment spread phenomenon was the actual migration of melanocytes out from the pigmented graft epidermis into the white epidermis (2). In 1970 these investigators confirmed, once again using the technique of grafting immunologically marked melanocytes, that when black skin is grafted to red skin, the blackening around the grafts is due to the migration of donor melanin-producing melanocytes into the host skin (5), although the mechanism by which the melanin-producing "black" melanocytes displace the pheomelanin-producing "red" melanocytes remains unclear.

Billingham and Silvers thus laid to rest the infective hypothesis of cellular transformation that Billingham and Medawar (7) and others had postulated earlier. It is now established that naturally occurring white skin in piebald animals[1] is devoid of melanocytes and that the spread of pigment from graft to donor site is attributable to the movement of viable melanocytes from the grafts into the previously melanocyte-free white host epidermis (8).

In 1972 Orentreich and Selmanowitz (9) reported that pigment spread occurred in adult human epidermis. They placed pigmented skin autografts in leukodermic sites in a black woman with a long-standing depigmented scar caused by a chemical burn and noted a spread of pigment, confined to a 1 mm wide zone, around each autograft. Similar repigmentation around graft sites has since been observed in patients with hereditary piebaldism (10) and in one patient with quiescent vitiligo (11).

Beyond establishing that the event underlying pigment spread is the spread of melanocytes from pigmented graft to unpigmented skin, none of these investigators have tried to identify those factors which might influence the extent and direction of melanocyte movement. Only one published report addresses this question: in 1953 Fábián (14) tried to determine whether cutaneous nerves inhibit pigment cell migration. He placed black autografts on white guinea pig trunk skin and then severed

1. In piebaldism, or heritable white spotting, there is an absence of melanocytes in the epidermis of the unpigmented region (12). The developmental origin of piebaldism may lie in a defect in the timing of melanoblast migration from the neural crest, or in a hostility in the local tissue environment to melanoblast migration, differentiation or survival. In a recent interesting study of pigment cell migration in embryonic axolotls, Keller et al. demonstrate that the failure of melanophores to migrate in these amphibians is due to an inhibition or lack of support by the epidermis (13).

the cutaneous nerve supply around half of the grafts. Comparing the area of pigment spread around the denervated grafts to the area of those grafts whose nerve supply was uninterrupted, he found that denervation significantly enhanced the area of pigment spread.

Further Aspects of Melanocyte Migration In Vivo

The melanocytes of hair and skin originate in the neural crest and migrate to the epidermis early in embryonic development. This embryonic migration was first elucidated by Rawls in 1947 (1) and its mechanism has since been examined in some detail. In 1967 Mayer, using tritium-labelling techniques, followed the migration of melanoblasts from neural crest to peripheral epidermis in both mouse and chick embryos (15).

The migration of melanocytes in fully differentiated adult tissue was investigated in a transparent in vivo system by Henkind and Jay (16). They followed the movement of melanocytes from the conjunctival limbus of guinea pig eyes into the basal layer of the transparent corneal epithelium in repsonse to local irritation with colchicine; they observed no mitotic activity among the migrating melanocytes. Staricco has argued that repigmentation of abraded epidermis in the human scalp is accomplished by the proliferation and migration of melanocytes out from the outer root sheaths of hair follicles into the healing epidermis (17).

In a 1965 study Reams (18) examined the importance of population pressure in the migration of embryonic melanoblasts. He transplanted pieces of melanotic ectoderm from Campine chick embryos to the hind limb buds of white Leghorn chick embryo hosts, and defined the dosage of the melanoblast inoculum by the area of tissue grafted. He found that with

"large doses", the feathers as well as the skin of the recipient white Leghorn limbs became darkly pigmented, and the underlying muscles were laden with melanocytes, like a normal Campine embryo of comparable age. "Moderate doses" yielded pigmented feathers and skin but only incomplete pigment spread into the underlying limb muscles; and with "small doses" only a limited area of skin became pigmented, there were few if any pigmented feathers and no melanocytes at all to be found in the underlying muscle tissue. Reams identifed three conditions as critical to the migration of melanoblasts: 1) population pressure -- the donor tissue must contain adequate numbers of precursor pigment cells to establish the critical population density required for emigration; 2) receptiveness of the tissue substrate -- differentiation cannot be so advanced structurally or morphologically as to inhibit the melanocyte migration; and 3) temporal coordination -- the critical population density in the donor tissue must be attained prior to restrictive differentiation of the receptor tissue substrate. Reams' study raises the question of whether similar factors might influence the migration of melanocytes in adult skin.

EXPERIMENTAL DESIGN

To test whether local melanocyte population density influences the extent of pigment cell movement in fully differentiated tissue, autologous melanocyte cell suspensions of varying concentrations were inoculated into suction blisters raised on achromic trunk skin of piebald guinea pigs.

Inoculation grafting of cell suspensions is a technique well-suited to the study of the effects of cell concentration on pigment spread. The method of grafting utilized by previous investigators (2,5), in which whole pieces of epidermis were grafted to exposed dermis, fails to provide control over the concentration of melanocytes introduced into the grafts. The use of epidermal cell suspensions as the graft vehicle offers the opportunity to control the critical variable of melanocyte concentration[2].

Preliminary testing in the laboratory of Dr. Sidney N. Klaus indicated that the technique of inoculation grafting into epidermal blisters could successfully induce pigment spread in piebald guinea pigs. A blister is by definition a separation at the epidermal-dermal junction, and it is in the basal layer of the epidermis that melanocytes naturally reside. The suction method of blistering was chosen in preference over other methods because it promised the least trauma.

Epidermal cell suspension in five concentrations ranging from $2 \times 10^7$ cells/ml to $5 \times 10^5$ cells/ml were tested and the results compared.

2. Billingham and Silvers did make use of epidermal cell suspensions as the graft vehicle in one experiment (5). They held drops of cell suspension in place on the exposed dermis with a covering of vaseline but they made no effort to quantify the amount of cell suspension grafted, the concentration of their suspension or the number of cells infused into the recipient sites.

MATERIALS AND METHODS

Six juvenile piebald guinea pigs with black ears and mostly achromic trunks were selected. The animals, comprising three females and three males, were segregated by sex throughout the duration of the study. All grafts involved autologous cells. Preparation and placement of grafts were done under sterile conditions.

Preparation of Inocula: Pigment cell suspensions were prepared from ear skin. On the day prior to grafting, the pigmented ear was cleansed with 70% ETOH and the hair shaved with a knife. The guinea pig was anesthetized with ether and part of the ear was amputated with a scissors. The tissue was laid flat and the subcutaneous far scraped off. It was then placed, dermal-side down, in a solution of 0.25% trypsin in TC Tyrode's Solution (Mg and Ca free), with 100 μg/ml gentamycin sulfate added, and incubated overnight at $4^{o}C$. A dermal-epidermal separation was performed mechanically with forceps, the intact epidermal sheet was placed keratin-side down in phosphate buffered saline (PBS) and the basilar cells were gently dispersed with forceps. Cell concentrations were determined with a hemocytometer, and when necessary the suspension was diluted with PBS to achieve the desired concentrations. Trypan blue exclusion tests were carried out on several occasions to determine the viability of cells injected; viability ranged from eighty-five to ninety-one percent.

Epidermal cell suspensions were prepared in five concentrations: $2 \times 10^{7}$, $1 \times 10^{7}$, $5 \times 10^{6}$, $1 \times 10^{6}$ and $5 \times 10^{5}$ cells/ml. Of these cells, melanocytes comprised approximately five to ten percent and the remainder were keratinocytes (19).

Preparation of Grafts: On the morning of inoculation, the trunk of the animal was shaved with clippers and the remaining stubble was removed with topical application of a keratolytic agent (Nair) for 10-20 minutes. The animal was sedated with sodium pentabarbital (Nembutal), injected intraperitoneally. Suction blisters were raised on the back and flanks according to the method of Kiistala (20). The suction device, consisting of 3 cc syringe flanges (10 mm diameter at the base) and flexible plastic tubing attached to a vacuum pump, was attached to the animal. Suction pressure was maintained at 100-200 mm Hg for approximately sixty minutes. Suction was terminated when a bulla (or several smaller vesicles) extending to the inner periphery of the flange appeared at each blister site. Three to six suction blisters could be raised simultaneously.

The cell suspension was then injected via 25 gauge needles into each blister site. Inocula ranged from 0.02 ml to 0.05 ml; it was impossible to ascertain the exact amount which remained within any given blister due to leakage from the ruptured vesicles at the blister margins. The location of each graft and the concentration of each inoculation was recorded.

Grafts of varying concentrations were placed randomly on each animal's trunk and each graft concentration was tested on at least three and usually four of the six animals. These precautions were taken to minimize any variations in responsiveness among the animals and also to guard against any minor variations caused by local tissue factors at various locations along the dorsum.

A total of eleven grafts at 5 x $10^6$ cells/ml and ten grafts each at the other four concentrations were placed and observed for ninety days. Additional grafts were placed and observed until biopsy at 7,45 and 55 days.

Observations and Measurement of Pigment Spread: The animals were observed at two-week intervals: each graft was inspected for establishment of pigmentation and the areas of pigment spread were measured with a ruler. At the end of ninety days the animal was photographed, the photographs were projected on a screen and the magnified outlines of the grafts were traced onto paper; the area of each pigment graft was then determined using a radial planimeter. Planimetric measurement insured maximum accuracy of area comparisons of the irregular shape of most of the pigmented patches.

Histology: Epidermal shave biopsies of graft sites were obtained at 7, 45, 55 and 90 days post-grafting, and stained using dopa, FMS and for EM.

For dopa stained sections, split thickness grafts were incubated for 30 minutes in 2 N sodium bromide at 37°. After separating the epidermis from the dermis with paired needles, the epidermis was incubated in a solution containing 0.5 mg dehydroxyphenylanine (dopa) for three hours at 37°. The epidermis was then fixed in formalin, sonified for ten seconds, dehydrated, cleared and mounted on a glass slide for analysis by light microscopy. For Fontana-Masson silver (FMS) staining, which blackens melanin, sections were fixed in Karnovsky's fixative, vertically sectioned and stained according to the method of Masson (21).

For electron microscopy (EM), sections were incubated immediately after biopsy in Karnovsky's fixative (one-half strength) for two hours at $4^{o}$; post-fixed in 2% osmium tetroxide buffered with phosphate for one and one-half hours, and then dehydrated in ethanol solutions. They were then infiltrated with epon-propylene oxide and embedded in Spurr following partial polymerization in a $60^{o}$ oven overnight. Sections were cut with an LKB ultramicrotome equipped with a diamond knife and examined under a Phillips electron microscope.

For melanocyte counts of dopa-stained sections a $mm^{2}$ ocular grid and light microscopic magnification x 160 was used.

RESULTS

At the end of ninety days, grafts ranged in size from as small as 5 $mm^2$ to as large as 155 $mm^2$. The average graft area was 33 $mm^2$. The largest diameter of any graft at the end of ninety days was 16 mm, and the average diameter was 8.4 mm (Appendix I).

Of fifty-one inoculation grafts observed for ninety days, successful establishment of pigmentation was achieved in thirty-three cases (65%). The likelihood of establishment of pigmentation was directly correlated with the concentration of cells injected. All ten (100%) grafts at the highest concentration inoculated, $2 \times 10^7$ cells/ml, were successful, whereas only three of ten (30%) of the grafts at the two lowest concentrations, $1 \times 10^6$ cells/ml and $5 \times 10^5$ cells/ml, resulted in permanent pigmentation (Table I). Applying paired $t$ tests to this data, the correlation of graft success with concentration of inoculum was statistically significant, P ranging from $P<0.05$ to $P<0.01$.

The area of pigment spread achieved bore no significant relation to the cell concentrations of the inoculum (Table II). Statistical examination of the data, applying paired $t$ tests, indicated that local melanocyte population density in the original inocula had no significant influence on the extent of melanocyte migration, $P>0.50$. Thus, to the extent that our methodology permitted control over the melanocyte density within the grafts, we conclude that cell concentration was not a significant factor in the degree of pigment cell migration in the grafts.

Graft size did not correlate with any particular trunk location: large and small pigmented areas were to be found with approximately equal

Table I

Correlation of Inoculation Graft Cell Concentration to Graft Success

| Cell Concentration of Inoculum (cells/ml) | No. of Grafts Placed* | No. of Successful Grafts** | % Graft Success |
|---|---|---|---|
| $2 \times 10^7$ | 10 | 10 | 100% |
| $1 \times 10^7$ | 10 | 9 | 90% |
| $5 \times 10^6$ | 11 | 8 | 73% |
| $1 \times 10^6$ | 10 | 3 | 30% |
| $5 \times 10^5$ | 10 | 3 | 30% |

*Number of inoculation grafts placed at that concentration and observed for ninety days.

**A successful graft is defined as one in which grossly visible pigmentation appeared within and persisted over the full ninety-day observation period.

Grafts varied in the likelihood of successful establishment of pigment spread depending upon the concentration of the inoculum. $P<0.05$ to $P<0.01$.

TABLE II

Area and Diameter of Pigment Spread Achieved* in Inoculation Grafts, By Cell Concentration of Inoculum

| Concentration of Inoculum (cells/ml) | No. of Successful Grafts** | Greatest Area ($mm^2$) | Average Area ($mm^2$) | Greatest Diameter (mm) | Average Diameter (mm) |
|---|---|---|---|---|---|
| $2 \times 10^7$ | 10 | 57 $mm^2$ | 27 $mm^2$ | 14 mm | 8.3 mm |
| $1 \times 10^7$ | 9 | 155 | 49 | 16 | 9.7 |
| $5 \times 10^6$ | 8 | 88 | 36 | 15 | 10.0 |
| $1 \times 10^6$ | 3 | 7 | 5 | 3 | 2.3 |
| $5 \times 10^5$ | 3 | 59 | 23 | 12 | 6.2 |

* Area and diameter achieved at the end of the ninety-day observation period.

** A successful graft is defined as one in which grossly visible pigmentation appeared within and persisted over the full ninety-day observation period.

The difference between the areas and diameters of spread among the grafts by inoculation concentration was not significant. $P > 0.50$.

frequency along the anterior flanks as well as the back, and there was no difference between pigment spread achieved in the more rostral as compared with the more caudal areas.

The density of melanocytes within the grafts at ninety days was calculated and compared to the melanocyte density found in naturally pigmented guinea pig trunk skin. Melanocyte density was found to be comparable from graft to graft irrespective of the concentration of the inoculum. And the average density of melanocytes in the grafts was not significantly different from melanocyte density in naturally pigmented trunk skin (controls) (Table III). This is in marked contrast to the density of melanocytes in the donor ear tissue: in our ear controls the average melanocyte density was 1416 cells/$mm^2$ (SE 44.1); other investigators have reported guinea pig ear melanocyte density in the range of 793 to 1500 cells/$mm^2$ (22).

Graft Appearance - Macroscopic

The pigmentation of the mature grafts was without visible scarring and the pigment was distributed in the epidermal pattern characteristic of guinea pig skin. Guinea pig trunk skin is not smooth; rather, like the skin of other hair-bearing mammals it displays a "corrugated" pattern, complementary to that of the dermal papillae, of low flattened hills alternating with broad shallow valleys. In guinea pigs the hills represent epithelial thickenings, about half-again as thick as the epidermis of the valleys. The hills run at right angles to the slope of the dorso-ventrally oriented rows of hair follicles (23). The melanocytes in naturally pigmented guinea pig skin are concentrated at the

TABLE III

MELANOCYTE DENSITY IN EPIDERMAL PIGMENT GRAFTS

Biopsies at 90 Days - Dopa Stain

| Cell Concentration of Inoculum | Animal & Graft | Melanocytes/$mm^2$ MEAN | SD | SE | n |
|---|---|---|---|---|---|
| 2 x $10^7$ cells/ml | 13-3(1) | 138 | 57.9 | 18.0 | 10 |
| 2 x $10^7$ | 13-3(2) | 155.5 | 55.9 | 16.9 | 11 |
| 1 x $10^7$ | 13-4(2) | 186 | 44.7 | 14.2 | 10 |
| 1 x $10^7$ | 13-4(3) | 224 | 63.8 | 20.2 | 10 |
| 5 x $10^6$ | 13-4(7) | 166 | 49.2 | 15.6 | 10 |
| 5 x $10^6$ | 13-4(8) | 152.5 | 93.7 | 33.1 | 8 |
| 1 x $10^6$ | 13-2(1) | 253.9 | 29.4 | 13.1 | 5 |
| Controls* | | | | | |
| 1 | 13-7 | 211 | 50.9 | 22.8 | 5 |
| 2 | 13-1 | 319 | 66.1 | 29.7 | 5 |
| 3 | 14-2 | 202.9 | 46.3 | 20.7 | 5 |

*Biopsies of naturally pigmented guinea pig flank skin, taken from piebald guinea pigs of the same strain used in this study.

summits and along the sloping sides of the hills and are completely absent from the valley regions (24). This melanocyte distribution pattern is reflected macroscopically in the corrugated appearance of the skin pigmentation (Figures 1,2). The pigment spread of the grafts conformed to this corrugated pattern: the striations of the epidermis were accentuated by the presence of pigment in the ridges and the apparent absence of pigment in the valleys (Figures 3,4,5). The hairs overlying the grafts were almost all white, although occasional black hairs could be seen. However, because of the infrequency of these black hairs and the otherwise uniform covering of white hairs, the grafts were barely visible unless the animal was shaved.

A number of grafts exhibited pigmentation, often but not always of the bluish dermal variety, for several weeks; the pigmentation then faded to invisibility over the rest of the observation period. The disappearance of these pigmented areas may have indicated melanocyte death, or severe dilution, or removal of melanotic debris from the dermis by phagocytes. (Such grafts were not counted as successful and the area of pigment spread achieved by them prior to their disappearance was not included in the data tabulations).

It was not unusual, especially in grafts which eventually achieved relatively large sizes, for more than one spot of pigment to appear at the graft site during the early weeks of the observation period. These multiply-pigmented grafts often were arranged in an annular fashion, and it was common for the pigment spread in these cases to proceed from the periphery inward as well as outward. In most of these cases, grafts which

displayed several pigmented patches early on subsequently coalesced to form large uniformly pigmented areas.

Unexpectedly we found that pigment spread reappeared at the sites of seven out of ten biopsies taken seven days post-grafting. In taking the shave biopsies we were careful to include the entire graft site and additional surrounding tissue, and most certainly took the full thickness of epidermis as well as considerable dermis; however, it is possible that the bases of some hair follicles were left behind. Some of the biopsied grafts achieved considerable size by the end of ninety days, with pigment spread up to 95 $mm^2$ (Appendix II).

Histology - Light Microscopy

Dopa-stained biopsy specimens taken at seven days post-grafting demonstrated sparsely distributed melanocytes in the epidermis (Figure 6). Most of these melanocytes were highly dendritic. A few dopa-positive cells with rounded cell bodies and very short or absent dendrites were seen. There were also occasional pairs of melanocytes with contiguous cytoplasm and few stubby dendrites, suggesting daughter cells viewed at the end of mitosis (Figure 7). The morphology of these cells is consistent with the careful description of mitotic melanocytes reported by Rosdahl and Lindström (25). They found that retraction of dendrites occurred during prophase so that metaphase cells were almost spherical in shape; signs of new dendritic growth emerged in late telophase and fully developed dendrites reappeared only after division was completed. Confirming the findings of other investigators (26,27), they reported that melanocytes remained dopa-positive and contained melanosomes during

all phases of mitosis. The fact that no mitotic figures were actually observed may reflect the limitations of the dopa stain technique employed. However, the dopa-positivity of these cells indicate that they represent functioning melanocytes.

Fontana-Masson silver (FMS) stained vertical sections at seven days post-grafting revealed normal guinea pig epidermis without evidence of inflammation or edema. Considerable amounts of extracellular melanotic debris were seen in the dermis (Figures 8,9). A few dendritic melanin-positive cells, probably melanocytes, were seen scattered in the dermis. In the basal layer of epidermis were rare clear cells. Occasional keratinocytes containing melanin granules were also seen in the epidermis. Although the normal ridge and valley pattern of trunk epidermis was evident no arrangement of pigment granules according to epidermal location was visible at this time.

FMS sections of grafts taken at 45 and 55 days showed a clearing of melanotic debris from the dermis. Some melanin-positive material remained in the dermis, most of which had been taken up by dermal cells, probably macrophages. No dermal melanocytes were seen. The epidermal pattern of melanin distribution seen in FMS sections at this time and at ninety day were indistinguishable from normally pigmented guinea pig epidermis: melanin-positive material and cells were concentrated in the basal layer of the epidermis; melanin granules in keratinocytes in the upper strata could be seen; and the areas of pigmentation were clearly arrayed along the crests and sides of the hills with little or no melanin-positive material to be seen in the valleys of the epidermal striations (Figures 10,11).

Dopa-stained horizontal epidermal sections taken at ninety days revealed uniformly distributed dendritic melanocytes arayed in striations corresponding to the hills of the ridged epidermis (Figure 12). The melanocytes appeared to advance in groups rather than as single cells, although occasional lone melanocytes were seen at the periphery. Unpigmented skin adjacent to the pigment spread were totally devoid of dopa-positive cells (Figure 13). The melanocytes at the periphery of the pigmented areas were noted to be somewhat larger in size, more dendritic and with slightly larger dendritic domains than those melanocytes more centrally loated (Figures 14,15).

Pigmented hairs from the graft sites were plucked and examined under the light microscope. The distribution of pigment within these hairs was regular and comparable to that found within normally pigmented hairs.

Histology - Electron Microscopy

Electron microscopic (EM) examination of biopsies taken at 45 and 90 days revealed some striking variations from normal. The melanocytes were normal in appearance, showing the typical well-developed rough endoplasmic reticulum and Golgi complexes and melanosomes in all stages of development (28). The melanocytes were located in the basal layer of the epidermis but no melanocytes were seen in direct contact with the basement membrane. Each melanocyte was noted to have a thin layer of keratinocyte cytoplasm and cell membrane separating the melanocyte from making direct contact with the basement membrane (Figure 16).

An additional discrepancy in the EM sections concerned the location of melanosomes within the keratinocytes. The size and density of distribution of the melanosomes within the keratinocytes was normal but their location was eccentric. Normally melanosomes are concentrated in a supranuclear position within the keratinocyte cytoplasm, but in the grafts the melanocytes were consistently concentrated in an infranuclear cytoplasmic distribution (Figure 17).

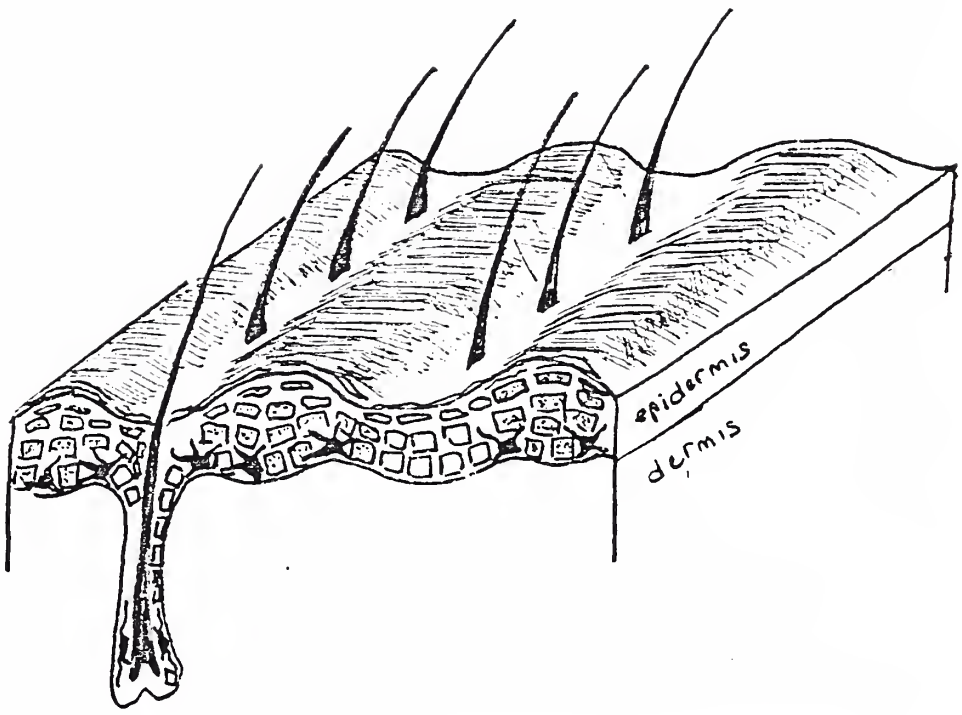



Fig. 1. Schematic representation of naturally pigmented guinea pig trunk epidermis, showing "corrugated" pattern and concentration of pigmentation in the hills.

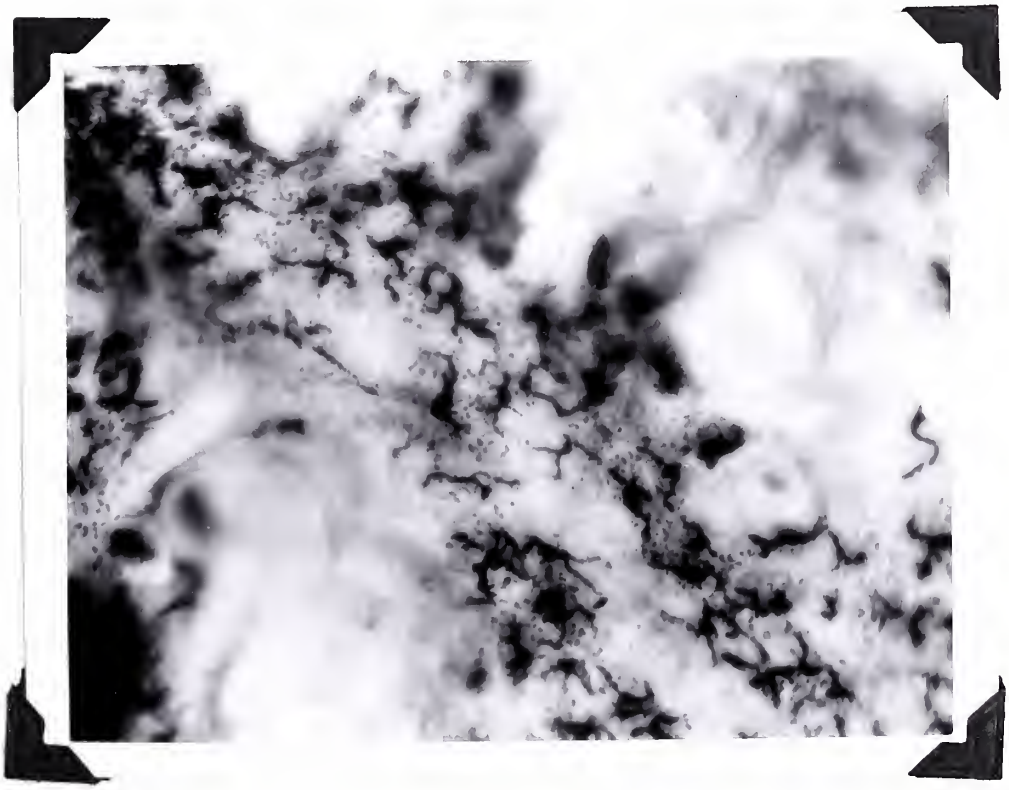


Fig. 2. Dopa stain of naturally pigmented guinea pig trunk skin, with melanocytes arrayed in rows corresponding to the hills of the epidermis. (x400)

Fig. 3 . Guinea pig with pigmented grafts at 90 days post-grafting. Note irregular shape of individual grafts. ( 1/2 actual size)

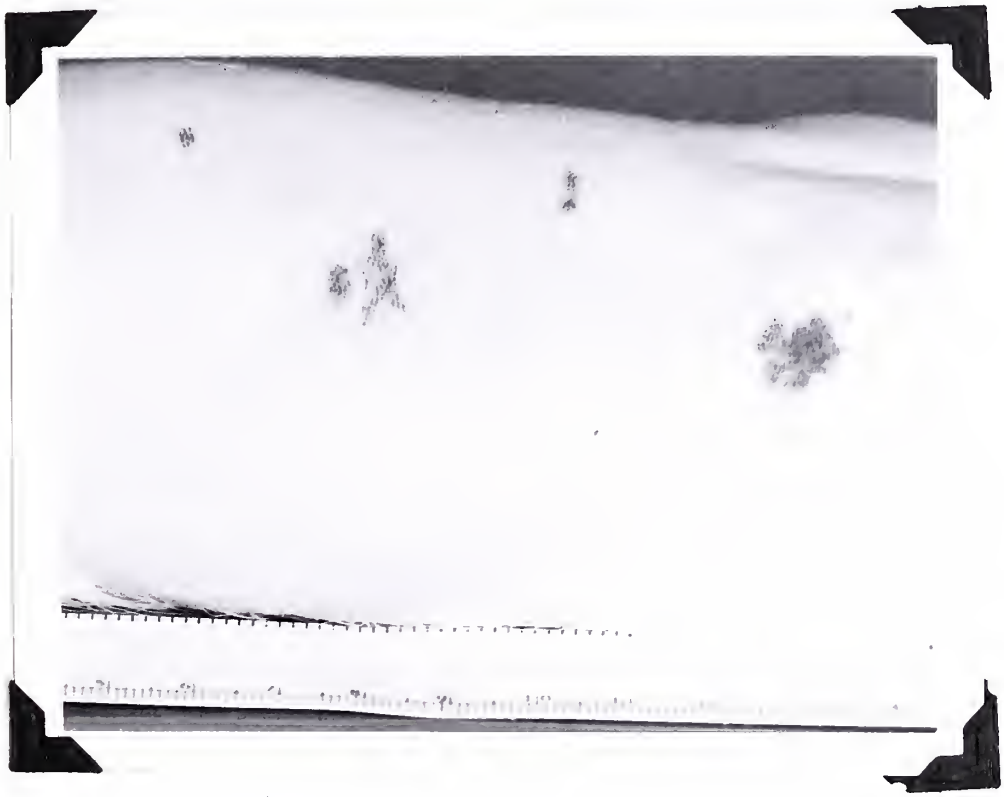


Fig. 4 . Close-up of animal shown in Fig. 3 . Note corrugated appearance of pigmentation. (1.5 x actual size)

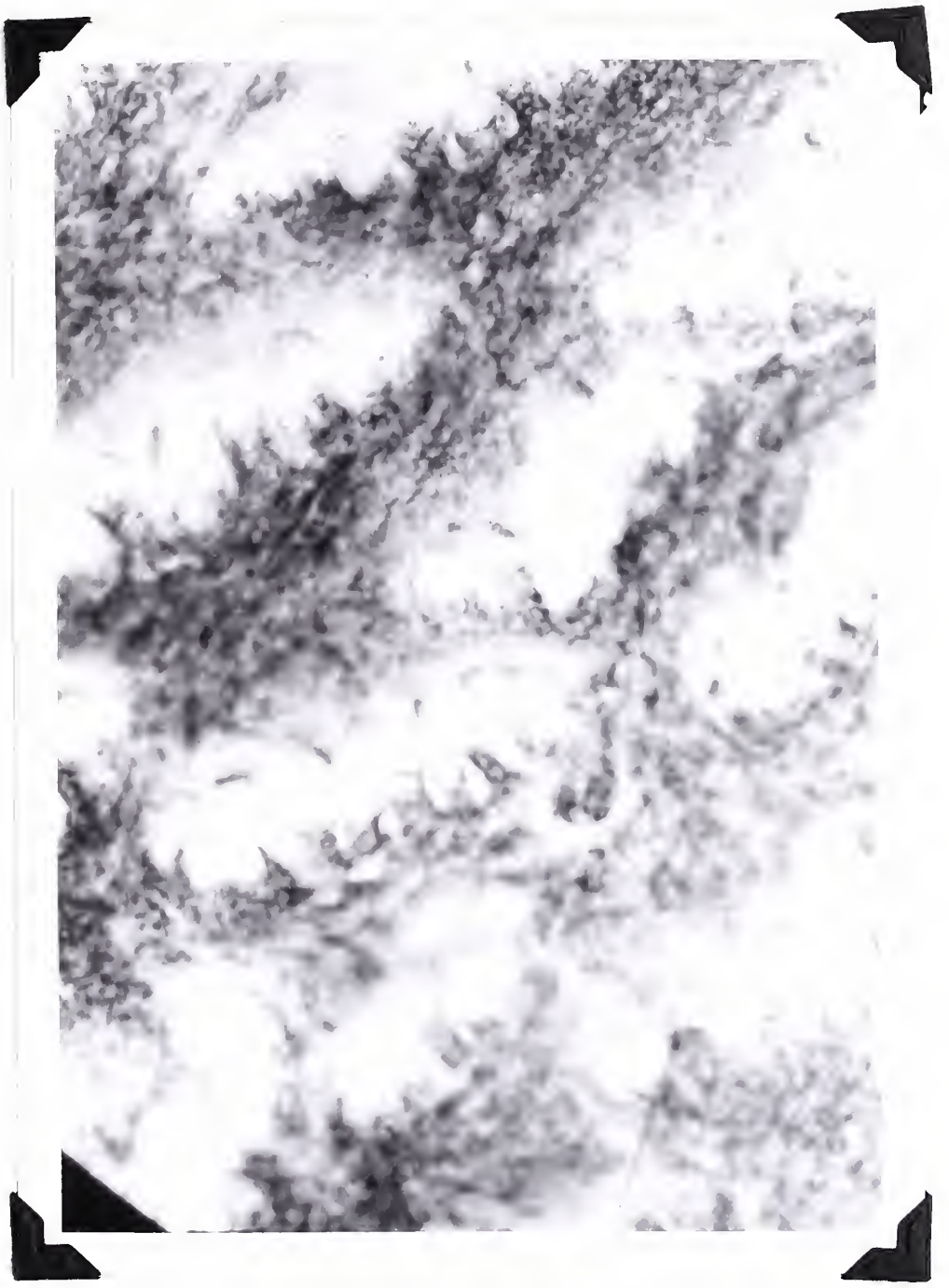


Fig. 5. Corrugated appearance of pigmentation in a graft at 55 days post-grafting. Unstained. (x40)

Fig. 6. Dendritic melanocytes in epidermis at 7 days post-grafting. Dopa stain. (x200)

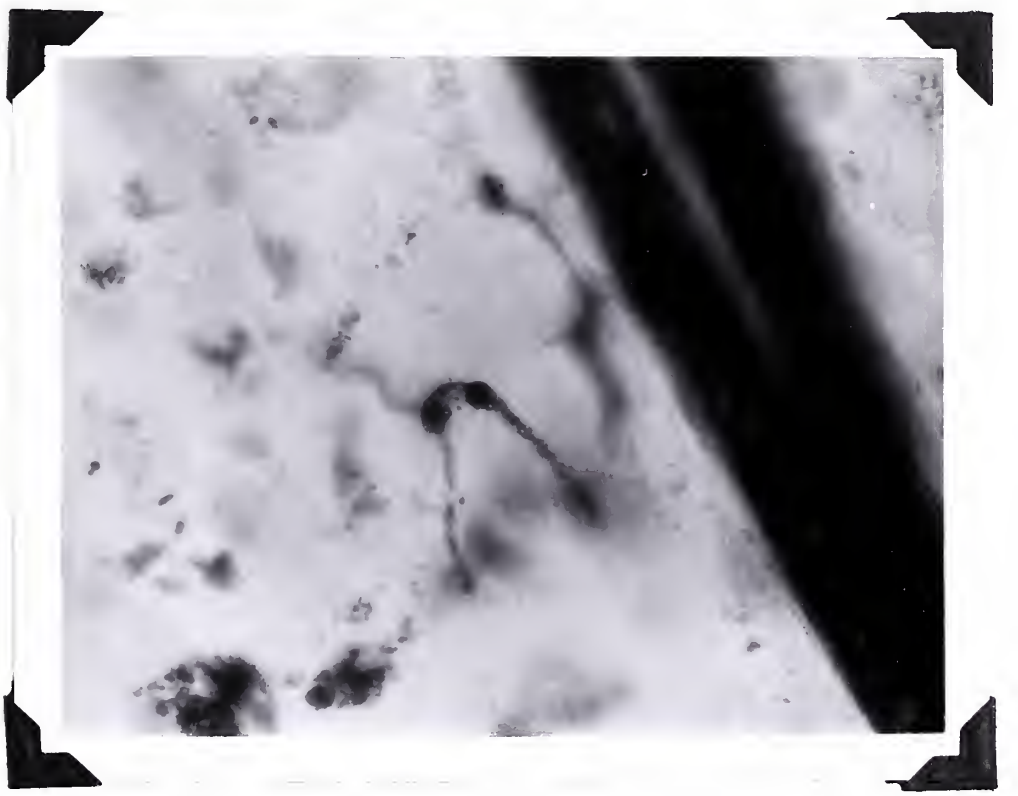


Fig. 7. Dopa-positive cells with contiguous cytoplasm, possibly daughter cells soon after cell division, in epidermis at 7 days post-grafting. Dopa stain. (x400)

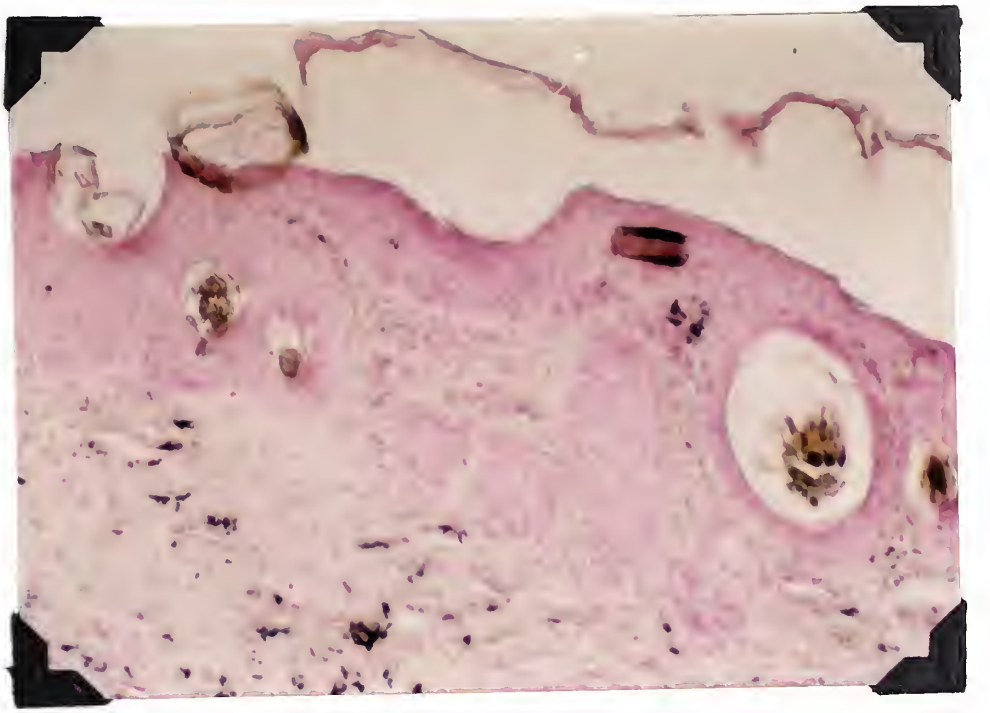


Fig. 8. At 7 days post-grafting melanocytes can be seen in the epidermis and in the dermis. Fontana Masson silver stain. (x 100)

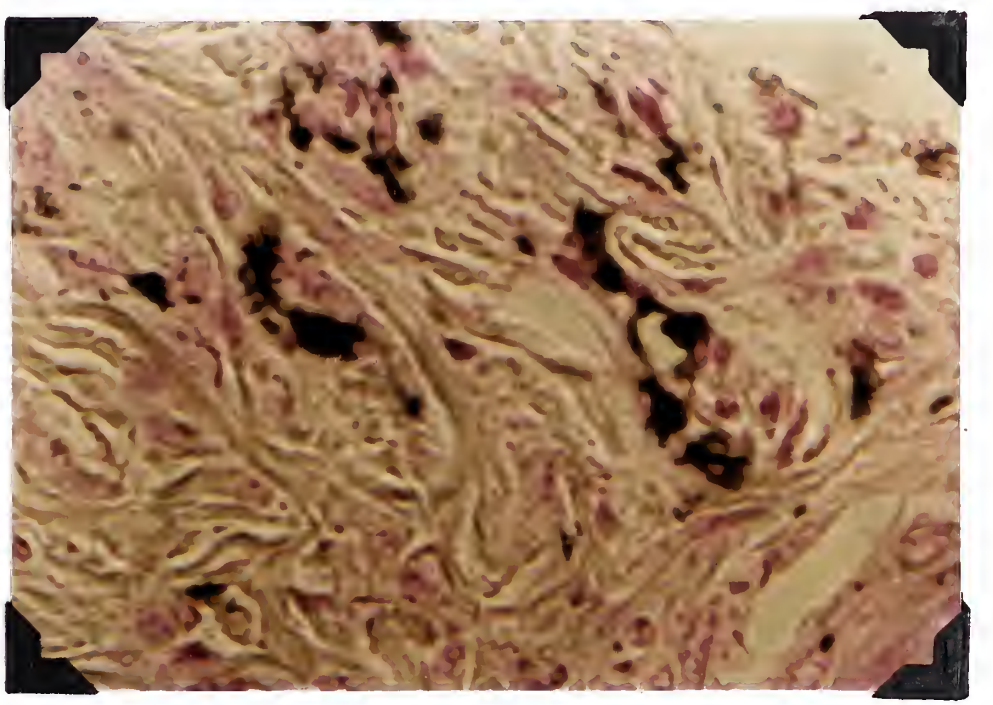


Fig. 9. Melanocytes and melanin-positive debris in dermis at 7 days post-grafting. Fontana Masson silver stain. (x 400)

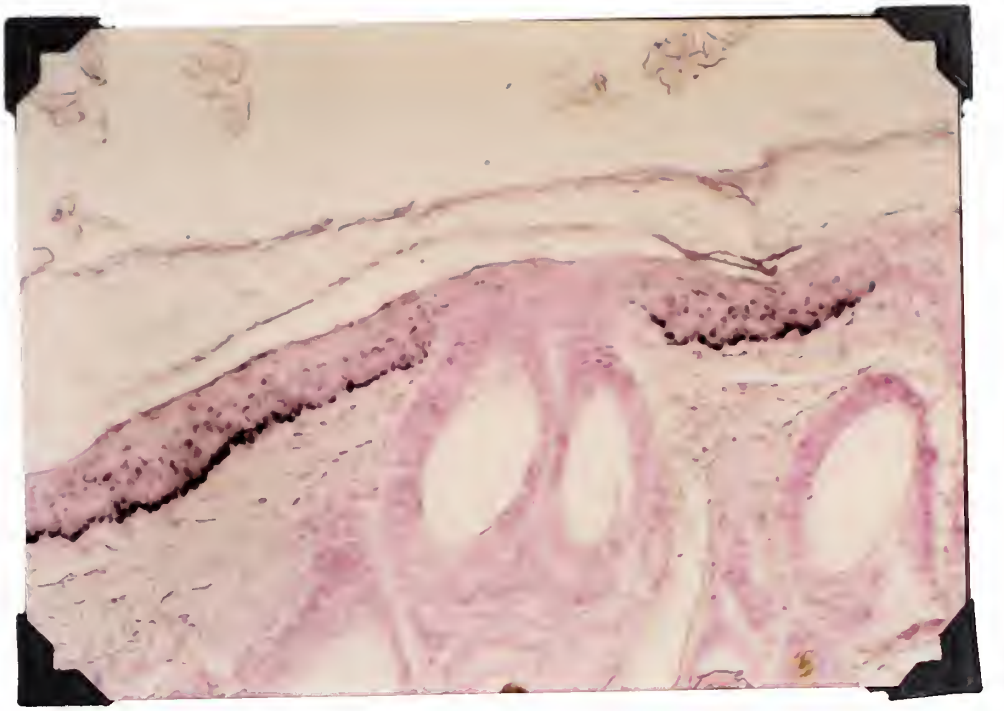


Fig. 10. At 55 days post-grafting the basal layer of the epidermis is densely melanin-positive except in the valley regions where the hair follicles reside. Fontana Masson silver stain. (x100)

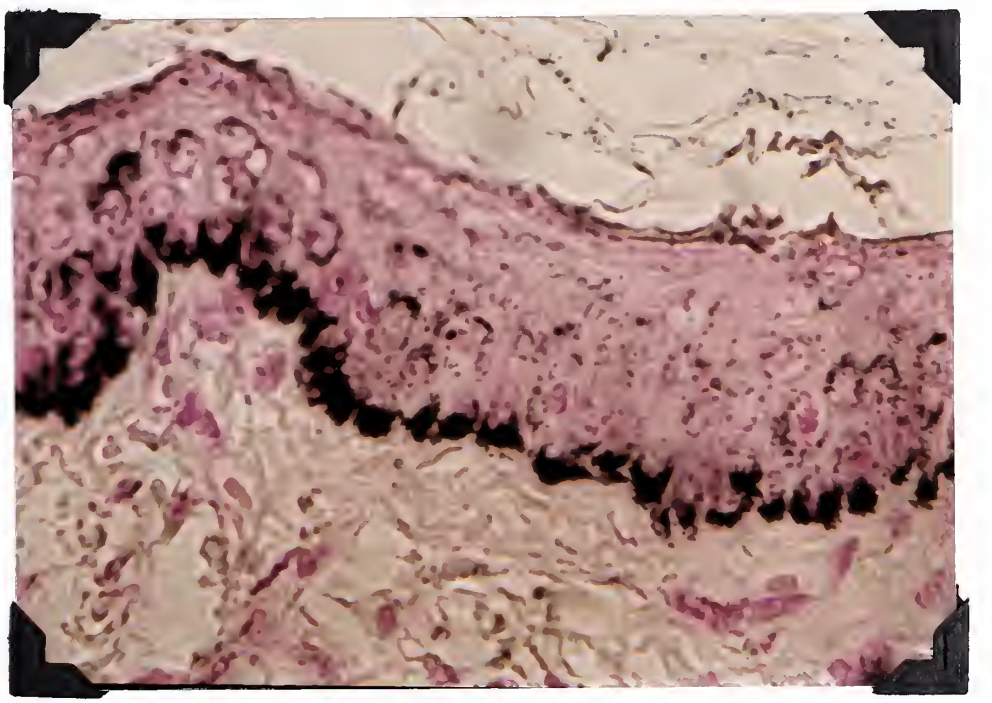


Fig. 11. Melanocytes and melanin-containing keratinocytes in the epidermis at 55 days post-grafting. Keratinocytes in the upper strata contain melanin-positive material. Fontana Masson silver stain. (x400)

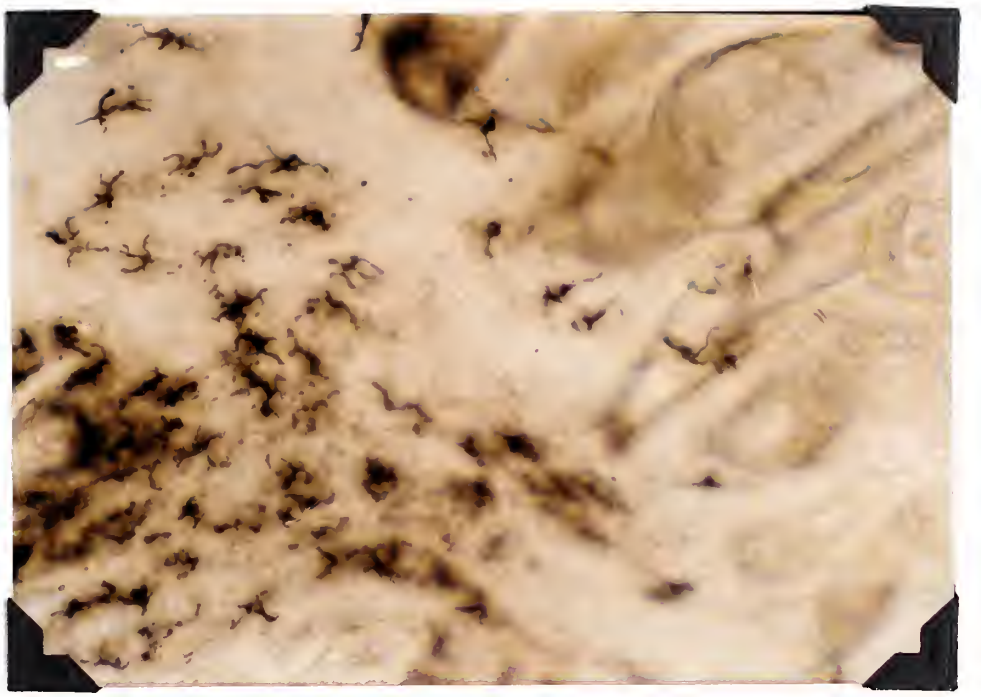


Fig. 12. Melanocytes in epidermis at 90 days post-grafting are distributed in striations corresponding to the hills of the epidermis. Dopa stain. (x200)

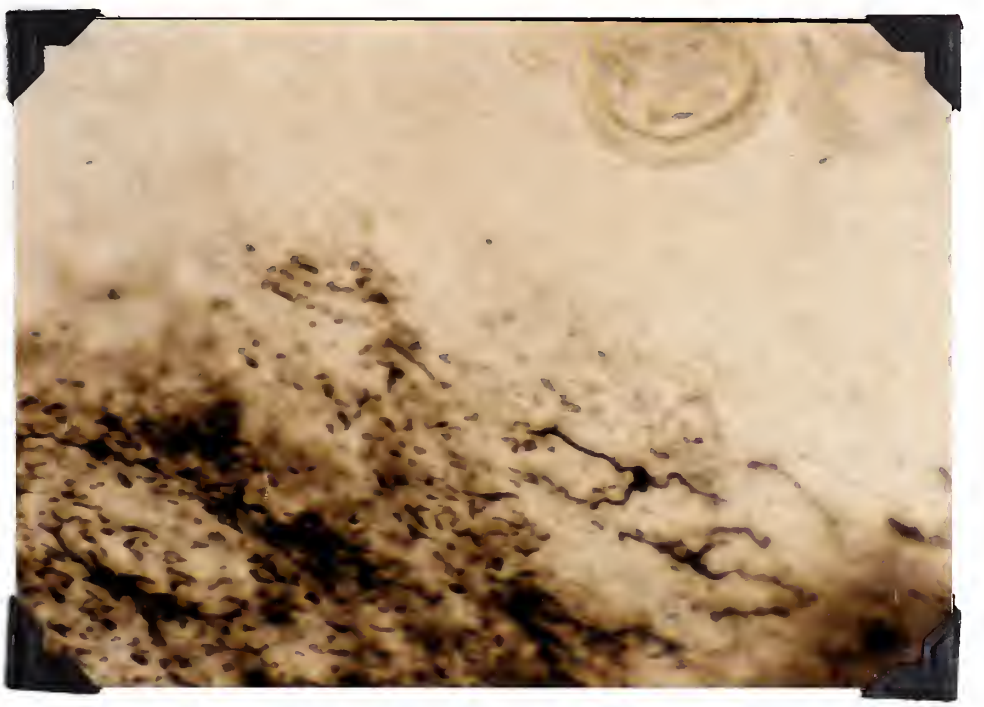


Fig. 13. Melanocytes at graft margin at 90 days post-grafting. Note the complete absence of pigment cells and pigmentation in the adjacent epidermis. Dopa stain. (x400)

Figure 14.

Camera Lucida drawings comparing melanocytes at the graft periphery to more centrally located melanocytes at 90 days. (x 400)

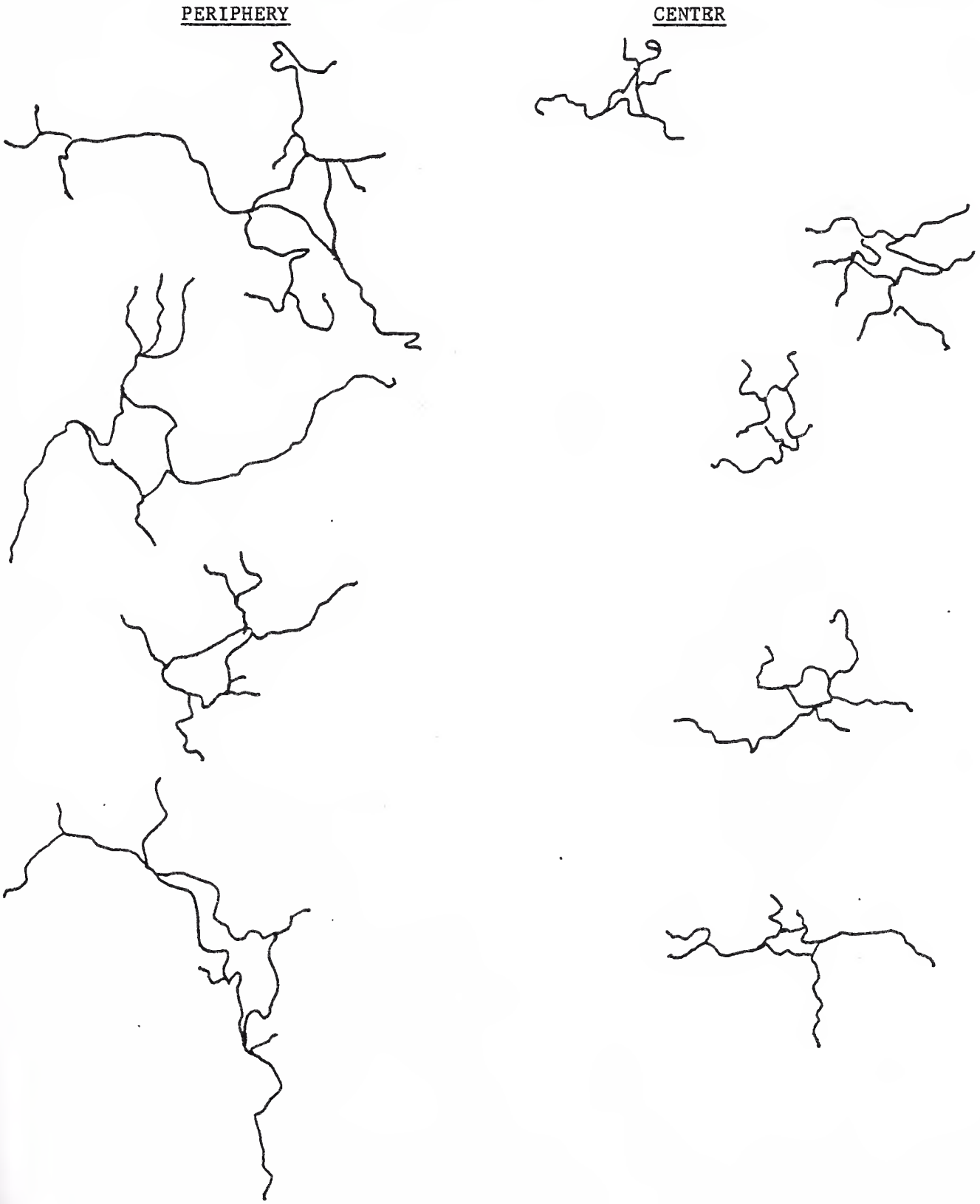
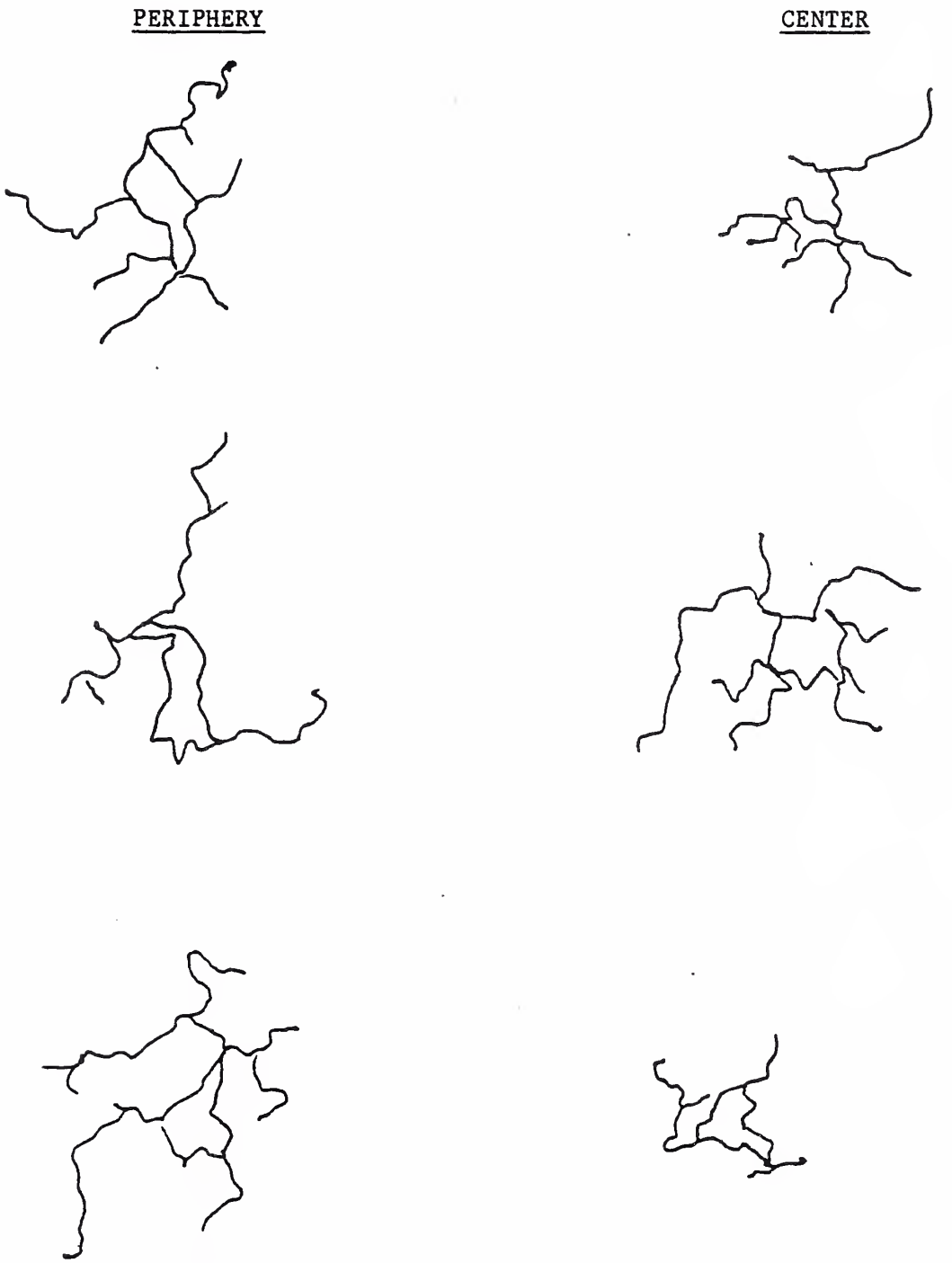


Figure 15.

Camera Lucida drawings comparing melanocytes at the graft periphery to more centrally located melanocytes at 90 days. (x 400)



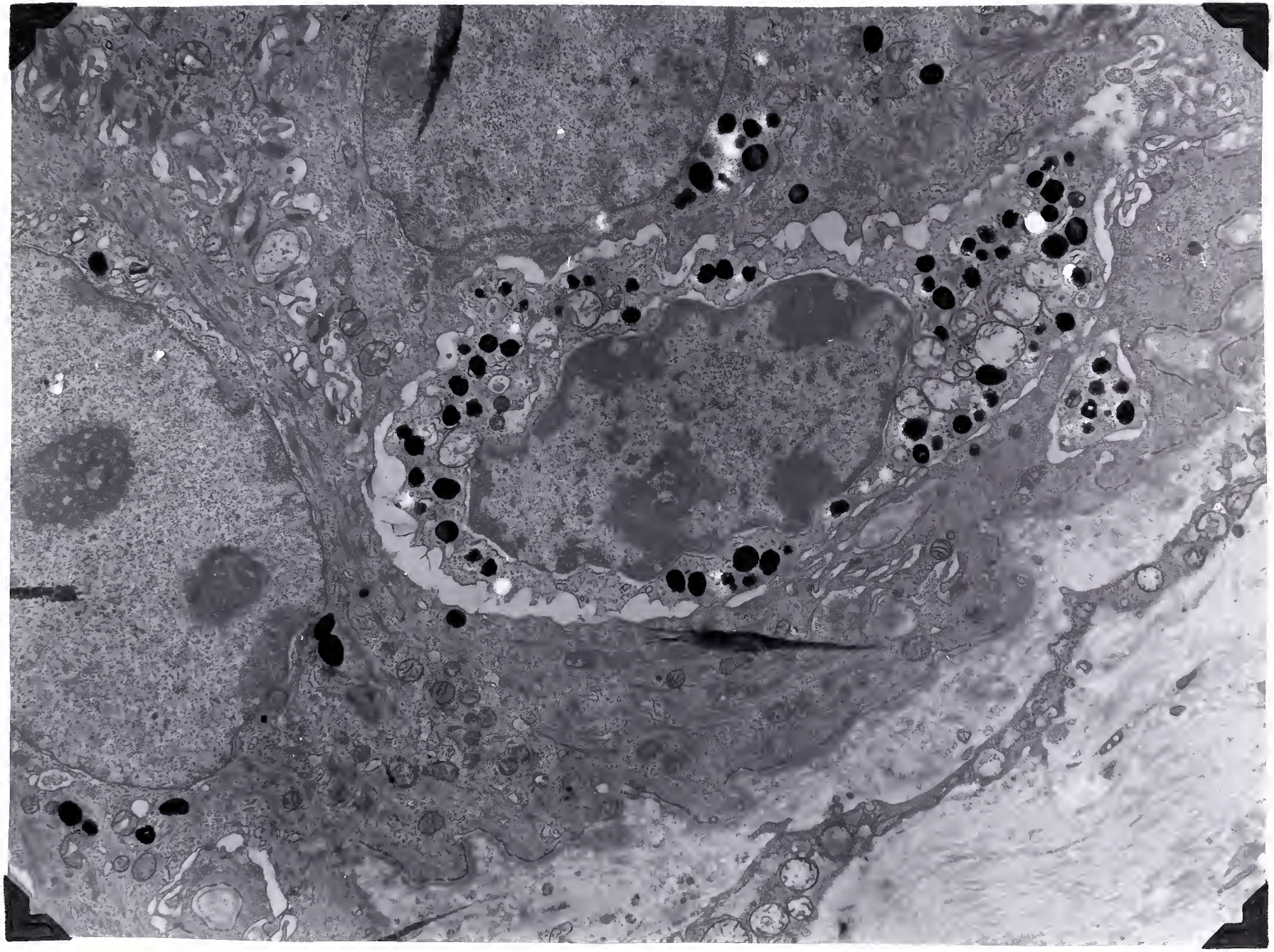


Fig. 16. Epidermal melanocyte at 90 days post-grafting is separated from the basement membrane by a tongue of keratinocyte cytoplasm. EM (x12,600)

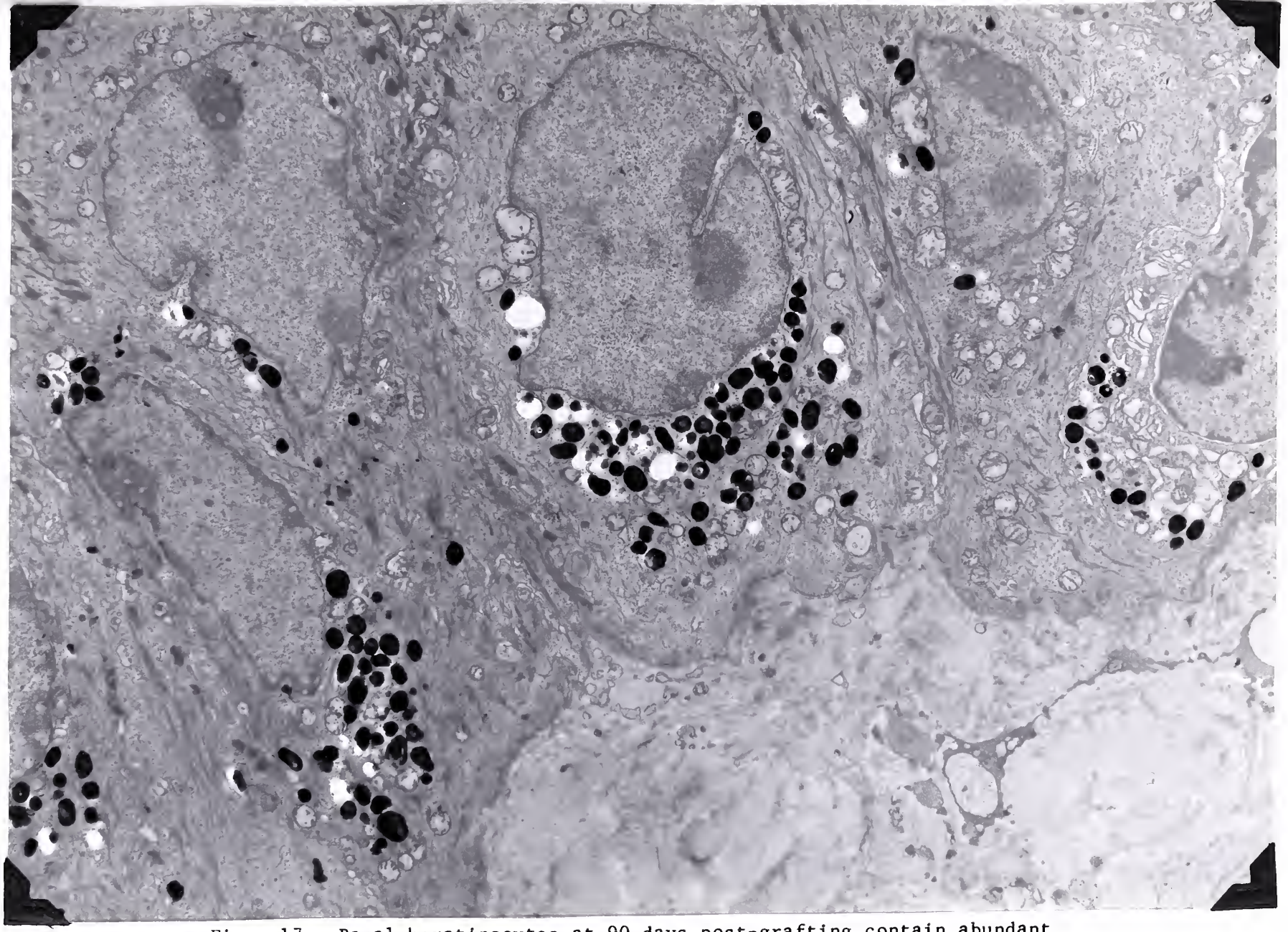


Fig. 17. Basal keratinocytes at 90 days post-grafting contain abundant melanosomes which are distributed in a predominantly infranuclear position. EM (x9200)

# DISCUSSION

## Methodology

This study was designed to investigate whether local melanocyte population pressure operates to enhance the degree of pigment spread in autografts placed on leukodermic skin. The somewhat novel methodology -- inoculation grafting of epidermal cell suspensions into suction blisters -- was selected because (1) it allowed for adjustment of melanocyte concentration within the grafts; (2) it promised the best possibilities for standardizing the size of grafts; and (3) it afforded the minimum of trauma to the host graft site.

Although it was not possible to quantify precisely the actual number of cells retained within each graft, the quantity of inocula ranged between 0.02 and 0.05 ml, so that it did not vary by more than a factor of 2.5. This difference could have obscured an influence of cell concentraion on area of spread between proximal concentrations, e.g. between $1 \times 10^7$ cells/ml and $5 \times 10^6$ cells/ml. But such a difference could not account for the lack of statistical difference in area of spread between more widely diverging concentrations: for example, between $2 \times 10^7$ cells/ml and $5 \times 10^5$ cells/ml, where the concentrations varied by a factor of 40. Furthermore, the very fact that we were able to demonstrate a statistically significant effect of cell concentration on the likelihood of establishment of pigment spread supports the relatively consistent standardization of our methodology.

Graft size was determined by the dimension of the suction flange (10 mm diameter) and the uniformity of suction pressure applied. According to

Kiistala (20), a suction bulla of standard size is obtained when flanges smaller than 15 mm in diameter are employed and suction is terminated when the epidermal separation has reached the inner circumference of the flange. Billingham and Silvers repeatedly noted their difficulties in standardizing the area of their grafts: they found considerable variance in the area of graft adherence when using epidermal split thickness grafts inserted on abraded recipient dermis (5,23).

Kiistala and Mustakallio (29) studied the histologic changes in human skin brought about by the suction blister technique. Grossly one sees minimal edema, no hemorrhage or petechiae, and, after a certain interval, the appearance of clear vesicles. Under light microscopy the blister roof is seen to comprise the entire epidermis, usually with the basal layer intact and retention of the normal epithelial columnar structure; the epidermis is separated from the dermis by a clear acellular space. Electron microscopic examination discloses that the separation occurs at the level of the lamina lucida, between the basal cell plasma membrane and the basal lamina. There was no evidence of damage to the integrity of the basal lamina. Our experience confirms the relatively atraumatic nature of the suction blister technique on guinea pig skin: H&E stained preparations of biopsied blister sites showed full separation of epidermis from dermis after one hour of suction at 115 mm Hg, although in our sections small amounts of clear fibrinous transudate and occasional erythrocytes were seen in the space separating epidermis from underlying dermis. Blisters healed rapidly, without evidence of scarring.

Pigment Spread and Cell Concentration

Our results indicate that initial melanocyte concentration in the grafts had no significant effect on the extent of pigment spread achieved, but that cell concentration was a significant factor in predicting the likelihood of establishment of pigmentation. Furthermore, once graft pigmentation was established, the density of melanocyte distribution within the pigmented areas was constant irrespective of the concentration of the inoculum.

How can we explain the absence of any discernible effect of graft melanocyte population density on the area of pigment spread achieved? One possibility is that cell density in the injected epidermal suspensions was not correlated with melanocyte survival in the grafts. Indeed, the artificially high densities in the higher concentrations tested may have had an adverse effect upon melanocyte survival post-inoculation; it would be interesting to test this question using cells labelled radioactively. However, our finding that graft cell concentration was directly correlated with successful pigment establishment suggests the contrary conclusion: that melanocyte survival within the grafts was related to increasing cell concentration.

Although his work was with embryonic rather than mature tissue and with chicks rather than mammals, Reams' work may shed some light on this question. He concluded that melanoblast migration was dependent on "(p)opulation pressure: the neural crest must contribute a sufficient number of initial precursor pigment cells which can actively proliferate to colonize the skin and establish the critical population density

required for emigration" (18 at 499, italics added). Reams quantified population by the area of the pigmented graft rather than by the number or density of melanoblasts within a particular graft; even so, he found significant differences in the extent of melanoblast migration depending upon the quantity of tissue grafted. Although Reams' data indicate that it may be necessary to introduce a critical number of melanocytes in order to achieve emigration from the graft site, his data do not support the conclusion that a critical cell density is a significant factor in melanocyte migration.

Our results are consistent with the notion that once a sufficient quantity of melanocytes is introduced into pigment-free but receptive host tissue, local factors rather than melanocyte density determine the extent of cell migration. Once pigment cells are established, the population density of melanocytes adjusts, such that the melanocyte density is the same from graft to graft, irrespective of initial inoculum concentration. And the melanocyte density of the grafts is comparable to the density of melanocytes in naturally pigmented guinea pig back skin rather than the much higher density found in the ear[3]. This strongly suggests that the determing factor or factors governing local melanocyte density within a given genotype does not originate in the melanocytes themselves, but rather in the local epidermis or underlying dermis.

Other investigators have reached a similar conclusion based upon the observed constancy of the ratio of melanocytes to keratinocytes in a particular region of skin in any given species (3). Although the number

3. Billingham and Silvers grafted pigmented ear skin on to white trunk skin. Although they do not discuss (and apparently made no attempt to calculate) the density of melanocytes in their areas of pigment spread, it is obvious from the photographs they present that the pigment spreading out from their grafts was less densely distributed than in the ear skin grafts themselves. See (2) at p. 354 and (23) at p. 29.

of melanocytes may vary slightly depending upon the extent of local stimulation, the pigment cell population is characteristic for a region of that genotype (18). In the human epidermis each melanocyte is associated with approximately thirty-six viable keratinocytes (30), while in the guinea pig the average melanocyte is associated with approximately twenty keratinocytes. Together a melanocyte and the keratinocytes in proximity to its dendritic processes comprise an "epidermal unit". There is no evidence that sex affects the distribution or frequency of melanocytes in mammals (23).

Why is the ratio of melanocytes to keratinocytes in a given region of a species so constant? Reams has hypothesized that "a given area is capable of supporting only a limited pigment cell population" (18). Cohen and Szabo suggest that epidermes vary in their power to influence the evocation of melanocytes from a local melanoblast population (3). Some sort of feedback control may occur, whereby the metabolic activity of the keratinocyte is altered by the arrival of melanosomes, and the affected keratinocyte then releases a chemical signal which regulates the level of melanocyte synthesis and transfer of melanosomes (8). Such a feedback mechanism might also influence the mitotic activity of melanocytes. Others have postulated that melanocyte and keratinocyte replication are regulated by "chalones" whose origin may be either in the epidermis or in the underlying dermis (31).

Lindstrom and Rosdahl (32) have proposed that repulsion between melanocytes is a factor in the density of their distribution, although they acknowledge that some sort of local signal originating in the

keratinocytes may also play a role. Using computer models and comparing melanocyte array in the mouse ear to the randomization predicted by a Poisson distribution, they found that the dendritic domains of the melanocytes varied inversely with melanocyte density; and that the dendritic trees of the melanocytes were oriented away from the cell bodies of close neighboring melanocytes (Figure 18). Although repulsion is an unusual property among like cells, these authors speculate that in this instance mutual repulsion might be biologically purposeful, serving to ensure an even distribution of pigment throughout the skin.

Melanocyte Proliferation

The pigment spread we observed almost certainly results from melanocyte proliferation as well as establishment and survival of inoculated cells. This can be demonstrated arithmetically: for example, graft 13-3(16), with an initial inoculum concentration of $5 \times 10^5$ cells/ml, achieved an area of 59 $mm^2$ in ninety days. Assuming that as many as 10% of the injected cells were melanocytes, that the viability of these melanocytes was as high as 90%, and that the quantity of the inoculum was, again taking the highest possible figure, 0.05 ml, we have: $5 \times 10^5$ cells/ml x 0.05 ml x 0.9 viability x 0.10 melanocytes = $2.3 \times 10^3$ melanocytes injected. Comparing this number with the number of melanocytes in the pigmented area at 90 days, 59 $mm^2$ x 182 melanocytes/$mm^2$ = $1.1 \times 10^4$ melanocytes, we see that the number of melanocytes in the graft exceeded the maximal possible inoculation quantity by a factor of 4.8. The difference between number of injected melanocytes and observed melanocytes at 90 days can only be accounted for

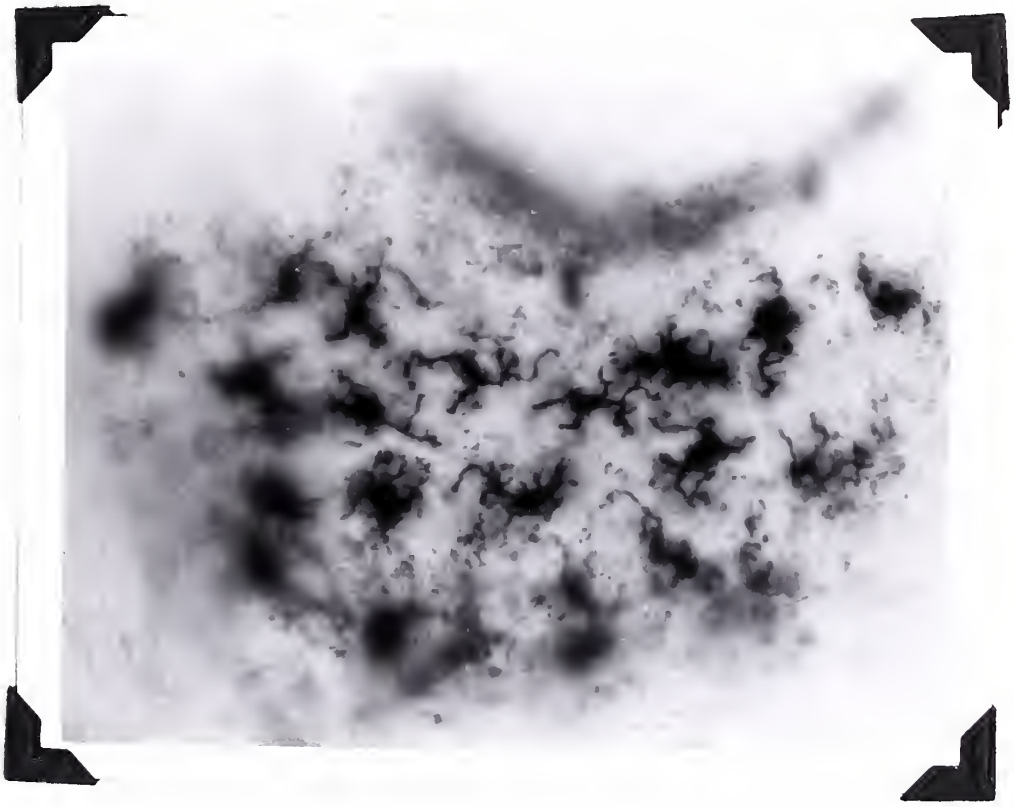


Fig. 18. Orderly distribution of melanocytes in epidermis at 90 days post-grafting. The dendritic trees of these cells are oriented away from the cell bodies of close neighboring melanocytes. Dopa stain. (x400)

by melanocyte proliferation. The dopa-positive cells of mitotic appearance seen in several biopsies further support melanocyte mitosis in the grafts.

For many years it was doubted that mature melanocytes were capable of mitosis. It is now well established by histologic and autoradiographic techniques that melanocytes divide in culture (3), in vivo in response to ultraviolet radiation[4] (33,34) and trauma (35,36), and also in unstimulated skin (37,33). The presence of such differentiating features as melanosomes and tryosinase does not preclude the mitosis of melanocytes under physiologic conditions, and the mitotic melanocytes do not lose these differentiating features during mitosis (25). While it is not known how many melanocytes are lost during hair and feather growth, mature melanocytes in the hair follicles divide during the hair growth phase (27).

However, the mitotic index[5] of epidermal melanocytes is far lower than that of keratinocytes. Jimbow et al. found that wax plucking of mouse skin increased the keratinocyte mitotic index by a factor of five to ten, but that even post-epilation the mitotic index of melanocytes was only 0.7% compared with 15% for keratinocytes (27). These investigators estimate that without stimulation the mitotic index of melanocytes may be as low as 0.1%. They point out that in the normal resting epidermis, there is little need for melanocyte replication since melanocytes, residing in the basal layer, are almost never shed while one keratinocyte is usually shed for each one generated.

4. The process by which skin exposed to UV light darkens ("tans") depends mainly on an increase in the synthesis and transfer of melanosomes rather than on an increase in melanocyte mitotic activity, although a slight increase in melanocyte mitosis probably does occur (27).

5. Mitotic index is defined as number of cells positive for uptake of tritiated thymidine divided by number of cells observed.

Our methodology may have served to stimulate the proliferation of melanocytes in the grafts. We shaved the animals with electric clippers every two weeks for the purpose of graft measurement, and whenever an animal was prepared for new grafting or photography its skin was epilated with Nair. It is interesting that Billingham and Silvers employed repeated similar hair removal over the sites of their grafts (2). Such procedures would be expected to enhance melanocyte mitotic activity, similar to the mitotic stimulation reported by Jimbow et al. with epilation of mouse hair (27). Staricco stimulated melanocyte mitosis in human scalp hair follicles by dermabrasion (17) and vibrapunture (38), and Giacometti et al. noted an increase in melanocyte proliferation in monkey scalp (36) and guinea pig (35) epidermis with superficial wounding. The use of trypsin to effect cell dispersion may also have stimulated mitotic activity: several investigators (39,40) have reported that trypsin activates cell division, although one study (41) suggests that trypsin may depress mitosis.

The unexpected appearance of pigment spread at the sites of biopsies taken seven days post grafting may be partly explained by the stimulating effect of trauma on proliferation of whatever melanocytes remained behind. Although it was our intention to do so, the full epidermal extent of the grafts may not have been excised; however, the size of the biopsies taken makes this explanation at best incomplete. Another possibility is that some of the injected melanocytes had by this time migrated into the bases of the hair follicles, some of which may have been left behind in the biopsy procedure. Staricco (17) found

melanocytes capable of mitosis and outward migration in the outer root sheaths of hair follicles, and Ortonne et al. (42) postulated a reservoir of melanocytes residing in hair follicles; however, these investigators were examining pigmented skin and there is no evidence that melanocytes preferentially migrate into hair follicles. Indeed, the relative sparseness of pigmented hairs in these grafts makes this explanation unlikely. The most likely location for these cells at that time may have been in the underlying dermis, only partly removed by the biopsy procedure; dermal melanocytes were observed in FMS stained sections at seven days post grafting. Migration of cells from dermis into epidermis can occur in various pathologic conditions such as inflammation and neoplasia (43). The extent to which intact basement membrane serves as a barrier to the passage of cells is not known, but the process of suction blistering may have disrupted basement membrane integrity, permitting migration of melanocytes from the dermis into the epidermis. Keratinocytes do exert an attracting influence on melanocytes *in vitro* (44), although whether such chemotaxis occurs *in vivo* in mature epidermis is not known.

Electron Microscopy

Light microscopic examination of the biopsied mature grafts indicated that the inoculated melanocytes had assumed their natural place and function in the healed host epidermis, but electron microscopy revealed two striking discrepancies from normal. First, although melanocytes located in the basal layer, they were never seen to make direct contact with the basement membrane: a fine layer of keratinocyte cytoplasm

appeared always to intervene. Second, although the keratinocytes appeared otherwise normal, and were normally attached to the basement membrane by hemidesmosomes, the melanosomes within these keratinocytes were consistently located in an infranuclear rather than supranuclear position.

Normally, melanocytes are regularly found in direct apposition to the basement membrane. The melanocyte-dermal junction is similar to the keratinocyte-dermal junction, although melanocytes lack true hemidesmosomal attachments to the basal lamina. Melanocytes have been noted to have hemi-desmosomal-like thickenings, termed "dense plates": these consist of a cytoplasmic dense zone abutting the internal leaflet of the plasma membrane with anchoring filaments extending from the outer leaflet of the plasma membrane through the lamina lucida to the basal lamina; however, the sub-basal dense plaques seen in the lamina lucida beneath true hemidesmosomes are absent from the melanocyte-dermal junction (43).

It is difficult to explain the failure of the grafted melanocytes to make direct contact with the basement membrane. The suction blister technique separates the epidermis from the dermis at the level of the lamina lucida, and the lamina lucida is known to be the location of certain glycoproteins -- specifically, laminin, fibronectin and noncollagenous bullous pemphigoid antigen -- which play a major role in the adhesion of cells to basement membrane (45,46,47). We might speculate that in the process of suction blistering, this adhesive matrix is briefly disrupted. If so, perhaps during the reconstitution of this

glycoprotein matrix the keratinocytes, by virtue of a greater affinity for these glycoproteins, were able to establish themselves in a more intimate relation to the basement membrane than the inoculated melanocytes. An alternative possibility is that trypsin may have temporarily modified the adhesive properties of the melanocytes. Trypsinization is known to alter cell surface adhesive properties (48), and trypsin may remain in treated cells in culture for as long as 48 hours (49). If trypsin did cause a temporary alteration in the adhesive properties of the grafted cells, such an alteration may have persisted long enough to give the local keratinocytes a temporal advantage in establishing their positions along the basement membrane.

The consistently inverted position of melanosomes within the keratinocytes is equally perplexing. Such a consistent eccentric location suggests some sort of fundamental disruption of cell polarity. The most likely cells to suffer from disrupted polarity would be the inoculated melanocytes and keratinocytes. We do not know whether the keratinocytes in the healed grafts derived from the trunk epidermis, either from the blister roof or the adjacent unblistered epidermis, or from the ear cell suspension inoculated. Some critical aspect of the polarity of the ear keratinocytes may have been disturbed by the dispersion process, which might be reflected in the eccentric position of their melanosomes. This hypothesis could be tested by radioactively labelling the cells of the ear suspension prior to grafting.

Clinical Considerations

Further understanding of the migratory behavior of melanocytes has

direct clinical significance to the treatment of a variety of hypopigmentary conditions. Selmanowitz (10) has demonstrated that pigmented autografts on achromic areas of adult human skin will result in pigment spread; however, he reported that punch grafts and split-thickness grafts resulted in scarring, and that transfer of blister roofs from suction blisters, although nonscarring, resulted in only small degrees of pigment spread. It is possible that the technique of inoculation grafting might someday provide repigmentation without scarring for patients suffering from hypopigmentary conditions.

## CONCLUSION

Inoculation grafts of epidermal cell suspensions were used to study melanocyte migration in juvenile piebald guinea pigs *in vivo*. We conclude that:

1. Inoculation grafts of epidermal cells produce permanent, normal-appearing pigmented spots on achromic guinea pig skin;
2. The proportion of successful grafts is directly correlated with the concentration of melanocytes inoculated; and
3. The melanocyte concentration of the inoculum is not a significant factor in the area of pigment spread achieved.

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# APPENDIX I

## Area and Diameter of Inoculation Grafts at 90 Days

| Concentration of Inoculum | Animal & Graft | Area($mm^2$) (manual) | Area($mm^2$) (planimetry) | Diameter (mm) |
|---|---|---|---|---|
| $2 \times 10^7$ cells/ml | 13-3(1) | 39 | 33 | 8 |
| | 13-3(2) | 35 | 27 | 10 |
| | 13-3(3) | 38 | 22 | 9 |
| | 13-6(6) | 35 | 19 | 8 |
| | 13-7(6) | 101 | 57 | 14 |
| | 13-7(7) | 20 | 16 | 6 |
| | 13-2(10) | 13 | 15 | 5 |
| | 13-2(11) | 62 | 37 | 11 |
| | 13-2(12) | 23 | 28 | 6 |
| | 13-2(13) | 33 | 15 | 6 |
| $1 \times 10^7$ cells/ml | 13-3(6) | 5 | 5 | 3 |
| | 13-3(7) | 67 | 47 | 11 |
| | 13-3(8) | 15 | 12 | 6 |
| | 13-4(1) | 20 | 13 | 7 |
| | 13-4(2) | 28 | 26 | 16 |
| | 13-4(3) | 41 | 27 | 9 |
| | 13-6(3) | 82 | 120 | 12 |
| | 13-5(12) | 178 | 155 | 16 |
| | 13-5(13) | 45 | 33 | 8 |
| | 13-5(14) | -- | -- | -- |
| $5 \times 10^6$ cells/ml | 13-2(6) | -- | -- | -- |
| | 13-3(9) | 13 | 10 | 5 |
| | 13-3(10) | 23 | 10 | 7 |
| | 13-3(11) | -- | -- | -- |
| | 13-4(6) | 10 | 18 | 4 |
| | 13-4(7) | 108 | 88 | 15 |
| | 13-4(8) | 29 | 33 | 7 |
| | 13-5(8) | 111 | 74 | 15 |
| | 13-5(9) | 7 | 8 | 3 |
| | 13-5(10) | -- | -- | -- |
| | 13-5(15) | 76 | 34 | 13 |

APPENDIX I (continued)

Area and Diameter of Inoculation Grafts at 90 Days

| Concentration of Inoculum | Animal & Graft | Area ($mm^2$) (manual) | Area ($mm^2$) (planimetry) | Diameter (mm) |
|---|---|---|---|---|
| $1 \times 10^6$ cells/ml | 13-2(8) | -- | -- | -- |
| | 13-5(1) | 2 | -- | 2 |
| | 13-5(2) | -- | -- | -- |
| | 13-5(3) | 5 | -- | 3 |
| | 13-2(1) | 7 | 3 | 2 |
| | 13-2(2) | -- | -- | -- |
| | 13-2(7) | -- | -- | -- |
| | 13-6(9) | -- | -- | -- |
| | 13-3(13) | -- | -- | -- |
| | 13-3(14) | -- | -- | -- |
| $5 \times 10^5$ cells/ml | 13-4(9) | -- | -- | -- |
| | 13-4(10) | -- | -- | -- |
| | 13-4(11) | -- | -- | -- |
| | 13-6(4) | 10 | 8 | 5 |
| | 13-6(5) | -- | -- | -- |
| | 13-7(5) | 2 | -- | 2 |
| | 13-7(10) | -- | -- | -- |
| | 13-3(12) | -- | -- | -- |
| | 13-3(15) | -- | -- | -- |
| | 13-3(16) | 87 | 59 | 12 |

# APPENDIX II

## Inoculation Grafts Biopsied at 7 Days: Area and Diameter Achieved at 84 Days Post Biopsy

| Concentration of Inoculum | Animal & Graft | Area ($mm^2$) (manual) | Diameter (mm) |
|---|---|---|---|
| $2 \times 10^7$ cells/ml | 13-6(1) | 48 | 7 |
| $2 \times 10^7$ | 13-6(2) | 95 | 11 |
| $1 \times 10^7$ | 13-6(7) | 50 | 8 |
| $1 \times 10^7$ | 13-6(8) | 88 | 12 |
| $5 \times 10^6$ | 13-7(9) | -- | -- |
| $5 \times 10^6$ | 13-7(8) | -- | -- |
| $1 \times 10^6$ | 13-7(1) | 35 | 9 |
| $1 \times 10^6$ | 13-7(2) | 51 | 9 |
| $5 \times 10^5$ | 13-7(3) | 29 | 7 |
| $5 \times 10^5$ | 13-7(4) | -- | -- |

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